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Lipid extrudates as novel sustained release systems for pharmaceutical proteins

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ABSTRACT

In order to develop improved lipid-based implants for proteins, the applicability of twin screw extrusion as a manufacturing strategy was investigated. Using lipid blends of low and high melting lipids, extrusion could be performed at moderate temperatures. In addition to the lipids, the implant systems contained 10% rh-interferon α-2a (IFN-α) co-lyophilised with hydroxypropyl-β-cyclodextrin (HP-β-CD), and 10% or 20% polyethylene glycol (PEG), respectively. Protein integrity was analysed by SDS-PAGE after extraction. FTIR-spectroscopy was used as a non-invasive method to assess the secondary protein structure of IFN-α within the lipid extrudates. Both methods indicate the preservation of the protein structure. Depending on the diameter of the prepared extrudates and on the PEG content of the system, protein release occurred in a sustained manner over 15, 40, or 60 days. Interestingly, extrudates with a smaller diameter revealed a continuous release profile, whereas extrudates with a diameter of 1.9 mm delivered IFN-α in a triphasic profile with a burst, lag and linear release phase lasting over 13 or 26 days.

In conclusion, lipid implants for proteins can be prepared by twin screw extrusion in an elegant and controllable manner that allows the adjustment of release characteristics.

1. Introduction

Despite the launch of several peptide-loaded microspheres and implants based on poly(D,L-lactic acid) (PLA) or poly(lactic-co-glycolic acid) (PLGA), no controlled release system for the delivery of proteins is on the market. Nutropin Depot®, the only protein loaded depot systems that reached commercialisation [1], was withdrawn from market by the manufacturers in 2004 [2]. The limited success of such systems can be explained by the fragile three-dimensional macromolecular structure of proteins which makes them susceptible to a variety of chemical and physical degradation pathways during manufacturing, storage, and release [3–6]. In particular, the frequently-used synthetic biodegradable polymers PLA and PLGA have several shortcomings. During the release period, for instance, the polymer matrix undergoes bulk erosion and reactive degradation products are trapped within the system. Consequently, the incorporated proteins are faced a completely altered microenvironment. In particular the significant pH drop [7], the increase of the osmotic pressure [8], and the accumulation of reactive species [9] have been identified as causes for protein unfolding, aggregation, and chemical degradation.

Apart from strategies for protein stabilisation [4,10] current research is focussed on the evaluation of alternative matrix formers. Physiological triglycerides, for example, can avoid many of the above mentioned disadvantages, suggesting their use as matrix material for peptides and proteins. Indeed, several authors showed that hydrophobic lipids processed either to lipid microspheres or to lipid implants represent a promising alternative for the controlled release of protein drugs (for review see [11–13]). For example, Mohl and Winter introduced a tristearin-based implant system for the continuous release of interferon α-2a (IFN-α) over 1 month [14]. Importantly, IFN-α was released almost exclusively in its monomeric form and co-lyophilisation with hydroxypropyl-β-cyclodextrin (HP-β-CD) provided long-term stability of these formulations [15]. Beside lipid implants based on tristearin, tripalmitin-based implants were developed for the sustained release of insulin [16], interleukin-18 [17], lysozyme [18], and brain-derived neurotropic factor [19].

However, the lipid implants described in these studies were prepared manually by compression with a handmade compression tool. Only two studies report protein-loaded lipidic implants that were prepared by other techniques. Pongjanyakul et al. filled polyethylene tubes (internal diameter 2.5 mm) with a suspension of lysozyme in molten glyceryl palmitostearate (Precirol® ATO 5). The tubes were closed and cooled. Afterwards, the solidified lipid was pushed out of the tubing and cut into implants with a length of 4 mm [20]. Yamagata et al. suggested that implants could be prepared by forcing a powder blend based on polyglycerol esters of fatty acids and solid IFN-α at elevated temperatures through a needle [21]. Obviously, all those methods for the manufacturing of lipidic implant systems rely on the preparation with a handmade manufacturing apparatus which is operated manually and only in lab-scale.

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In contrast, polymeric implant systems are often prepared by extrusion and in particular by screw extrusion [22,23]. The processability of lipid materials by such techniques has been demonstrated for the preparation of oral dosage form intermediates for low molecular weight drugs. In this context lipids can either be used as lipophilic binder or as matrix material [24–27]. For instance, Reitz and Kleinebudde prepared theophylline-loaded extrudates based on glyceryl palmitostearat or glyceryltrimyristate. However, the slowest release profiles featured a release of 80% theophylline within 40 h [26], which is a time span far away from what is aimed for the delivery of proteins.

Despite the potential advantages of screw extrusion, including the ease of scale-up as well as the flexibility with respect to implant geometry, studies on the potential of producing protein-loaded lipid implants by screw extrusion were not available. Therefore, it was the aim of the present study to develop and to evaluate extrusion protocols for the manufacturing of sustained release devices for proteins. Apart from the basic requirement for such a system that the lipid matrix should be present in the stable modification, the maintenance of the protein structure can be considered as the most daunting task during manufacturing and release. The major objectives of the present study thus include thorough investigation of the lipid modification and the protein stability after extrusion.

2. Materials and methods

2.1. Materials

The mono-acid triglycerides trilaurin (Dynasan D112), tripalmitin (Dynasan D116), tristearin (Dynasan D118), triarachidin (Dynasan D120) as well as the mixed acid triglycerides H12 and E85 were kindly provided by Sasol GmbH (Witten, Germany). The specifications of H12 and E85 provided by the manufacture are summarized in Table 1. Rh-interferon α-2a (IFN-α, Mw = 19.237 kDa, stock solution: 1.695 mg/mL in 25 mM acetate buffer pH 5.0, containing 120 mM sodium chloride) was kindly provided as a gift by Roche Diagnostics (Penzberg, Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Merck, Darmstadt, Germany and polyethylene glycol 6000 (PEG) from Clariant, Gendorf, Germany.

2.2. Preparation of lipidic extrudates

IFN-α was lyophilised with HP-β-CD in a protein/excipient ratio of 1 to 3 (wt). Before lyophilisation the pH of the stock solution was adjusted to 4.2 with acetic acid (30% vol/vol). HP-β-CD was used as lyoprotectant as it was shown before that HP-β-CD enabled an efficient protein stabilisation not only during freeze-drying [28] but also during long-term storage of compressed lipid implants [15]. A freeze-dryer ε12G (Christ, Osterode, Germany) was used for lyophilisation. A 1 mL aliquot of protein/lyoprotectant stock solution in 2R glass vials was frozen to −45 °C at a rate of 1.6 °C/min. This temperature was maintained for 90 min. Afterwards, the temperature was increased to −20 °C in steps of 0.1 °C/min. Simultaneously, the chamber pressure was reduced to 10^{-2} mbar. This primary drying program was maintained for 30 h. Then, the temperature was increased by 0.1 °C/min up to 20 °C and concomitantly the pressure was reduced to 10^{-3} mbar. Secondary drying was performed for 15 h. Finally, the chamber was vented with nitrogen to a pressure of 800 mbar and the vials were closed with rubber stoppers.

Ten percent IFN-α/HP-β-CD lyophilisate and PEG, optionally 10% or 20%, were blended with the lipid powder comprising Dynasan D118 and the H12 in a mass ratio of 1 to 4. The obtained mixture was filled in the preheated twin screw extruder (MiniLab®Micro Rheology Compounder, Thermo Haake GmbH Karlsruhe, Germany) and extrusion was carried out at 40 °C with a screw rotation speed of 40 rpm. Extrusion was performed with closed bypass without circulation. In order to prepare extrudates of different sizes, dies with a diameter of 0.5 mm or 1.0 mm were fixed in front of the standard extruder outlet (diameter 2.0 mm). The extruded strands were cut into pieces with a length of approximately 2.3 cm.

Table 1
Characteristics of the used mixed-acid triglycerides H12 and E85 as provided by the manufacturer

Triglyceride type	H12	E 85
C12 fatty acid, %	71.0	27.0
C14 fatty acid, %	26.8	70.1
C16 fatty acid; %	2.0	2.0
Hydroxyl number (mg KOH/g)	1.4	3.1
Saponification no. (mg KOH/g)	269.38	240.5
unsaturated fatty acids	no	no
Melting point (°C)	35.5	41.3

2.3. Wide-angle X-ray scattering (WAXS)

Wide-angle X-ray scattering (WAXS) was performed by an X-ray Diffractometer XRD 3000 TT (Seifert, Ahrensberg, Germany), equipped with a copper anode (40 kV, 30 mA wavelength 0,154178 nm). Experiments were conducted at 0.05° (2 theta) within a 5°–40° range.

2.4. Differential scanning calorimetry (DSC)

Samples were ground and approximately 10 mg were analysed in a sealed Al-crucible. Measurements were performed with DSC 204 Phoenix (Netzsch, Selb, Germany). Heating and cooling were conducted at a scan rate of 5 K/min within a −20 to 100 °C temperature range.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein was extracted with the aqueous extraction method developed by Mohl [28]. Briefly, the protein-loaded matrix was ground in an agate mortar. Subsequently, 50 mg of the sample were suspended in 1 mL pH 7.4 isotonic 0.01 M sodium phosphate buffer containing 0.05% (wt/vol) sodium azide and 1% (wt/vol) polysorbate 20 (PBST). After gentle agitation for 2 h, the samples were centrifuged at 5000 rpm for 5 min (4 K15 laboratory centrifuge; Sigma, Osterode, Germany). SDS-PAGE was conducted under non-reducing conditions using an XCell II Mini cell system (Novex, San Diego, CA). The protein solutions were diluted in a pH 6.8 Tris buffer, containing 2% SDS and 2% glycerin. Samples were denatured at 90 °C for 30 min and subsequently 20 µL were loaded into the gel wells (NUPAGE Novex 10% Bis Pre-Cast Gel 1.0 mm; Invitrogen, Groningen, The Netherlands). Electrophoresis was performed in a constant current mode of 30 mA in a Tris–glycine/SDS running buffer (MES running buffer; Invitrogen, Groningen, The Netherlands). Gel staining and drying was accomplished with a silver staining kit (SilverXpress) and a drying system (DryEase), both provided from Invitrogen (Groningen, Netherlands).

2.6. In vitro release studies

The protein-loaded implants were placed into TopPac® vials (cycloolefin copolymer vials; Schott GmbH, Mainz, Germany) filled with isotonic 0.01 M phosphate buffer pH 7.4 containing 0.05% (w/v) sodium azide. For extrudates with a diameter of 0.5 or 1 mm 1.0 mL of buffer was added, whereas extrudates with a diameter of 1.9 mm were incubated in 2.0 mL buffer media. The vials were placed in a horizontal shaker (40 rpm, 37 °C, Certomat®IS; B. Braun Biotech International, Göttingen, Germany). At predetermined time points, the release medium was completely exchanged and the amounts of released IFN-α were determined by size-exclusion chromatography as previously described [14].

2.7. Fourier transform infrared spectroscopy (FTIR)

In order to investigate the secondary protein structure after extrusion IFN-α loaded extrudates were ground and 2 mg of the material were blended with 150 mg KBr. The mixture was compressed at 78.4 kN for 2 min. For comparison KBr pellets of the lyophilised IFN-α and 2 mg the lipid/protein/PEG blend before extrusion were prepared in the same manner. The KBr pellet was fixed in the sample holder (Tensor 27, Bruker Optik, Ettlingen, Germany) and spectra were collected with a total of 256 scans at a resolution of 2 cm^{-1}. The obtained absorbance spectra were automatically baseline corrected (OPUS, Bruker Optik, Ettlingen, Germany). Background correction was performed manually. The obtained spectra were vector-normalised and analysed by the second derivatisation in the amid I band region (OPUS, Bruker Optik, Ettlingen, Germany).

3. Results and discussion

3.1. Preparation of lipidic extrudates

Tristearin implants prepared by compression had previously been identified as a promising delivery platform for IFN-α [14,15]. Therefore, it was evaluated whether the processing of the corresponding implant formulation based on tristearin plus various amounts of polyethylene glycol 6000 (PEG) and the protein co-lyophilised with hydroxypropyl-β-cyclodextrin (HP-β-CD) can be realised by twin screw extrusion. With respect to the method used to adjust the viscosity, extrusion processes can be classified into molten systems (hot-melt extrusion) and semisolid systems. Semisolid systems are generated by dispersing a high portion of solid material in a liquid phase [23]. This technique is widely used to prepare granules or pellets, whereas for the preparation of parenteral controlled release devices mostly the hot melt extrusion technique is applied [22].

However, it is well-known that pharmaceutical proteins are only marginally stable at elevated temperatures. For example, for IFN-α 50% of the protein unfold at around 60 °C (T_m) when dissolved in acetate buffer (pH>4.0) [29]. In contrast to proteins in aqueous solution, proteins suspended as dry powders in non-aqueous media maintained their biological activity even at high temperatures. Solid proteins in anhydrous organic solvents retain their correct conformation and the minimal layer of water necessary to solvate folded proteins remains associated with the protein. Since unfolding would require a greater number of water molecules, unfolding becomes unlikely in hydrophobic systems. Moreover, water is a mandatory for reactions such as deamidation and hydrolysis. Thus, proteins can also be expected to be more stable against chemical degradation when they are suspended in organic solvents [30,31].

Assuming parallelism between non-aqueous protein suspensions and the dispersion of dry protein powder within a molten lipid high resistance of proteins against heat can be expected during extrudate manufacturing. Indeed, a few authors reported such scenarios. For instance, IFN-α dispersed in molten polyglycerol esters of fatty acids (60 °C) maintained 95% of its antiviral activity after incubation at 60 °C for 6 h [21]. Furthermore, the biological activity of lysozyme dispersed in molten glyceryl palmitostearate (65 °C) was not affected [20].

Based on the assumption of an increased thermal stability of IFN-α suspended in the molten or partly molten lipid matrix we tried to prepare implants based on the standard formulation (80% tristearin, 10% HP-β-CD placebo lyophilisate and 10% PEG) by heating the extruder barrel. However, tristearin exhibits a very sharp melting point and extrusion with various temperature programs failed. At extrusion temperatures below the melting point of tristearin filling of the extruder was not possible, whereas temperatures above the melting resulted in low viscosity melts inapplicable for extrusion.

In order to overcome these problems, various amounts of tristearin were replaced by alternative lipids with a lower melting point. Thereby, selective melting of the low melting point lipid should provide a softening of the lipidic mass during the manufacturing procedure which should enable the flow through the die. Furthermore, the use of low melting point lipids and the extrusion at low temperatures might be especially beneficial for the handling of temperature-sensitive drugs such as proteins. The mixed acid triglyceride H12, based on saturated, even-numbered, unbranched, natural fatty acids was chosen for tristearin replacement. H12 melts at 36 °C, thus the extruder was heated to 40 °C to ensure a complete melting of H12 during manufacture. The amount of H12 needed for acceptable extrusion was explored in a preliminary study. Initially, 40% H12 was applied, allowing the filling of the extruder. However, the obtained rods did not solidify after leaving the extruder outlet. By reducing the amount of the low melting point fat in steps stable extrudates could be produced. The optimum amount of H12 was found to be 16% (corresponding to a H12 to tristearin mass ratio of 1 to 4).

In order to evaluate if this formulation strategy is transferable to other lipid combinations the low- or high-melting point lipid was substituted. The triglyceride E85 was used instead of H12. E85 is also a mixed-acid triglyceride but, in comparison to H12, the amount of myristic acid is increased accounting for a melting point of 41 °C. Thus, extrusion was performed at a temperature of 45 °C. Like for H12, an addition of 16% E85 facilitated a continuous extrusion. In order to evaluate if the developed manufacturing procedure is also applicable for lipidic blends based solely on mono-acid triglycerides, H12 was replaced by trilaurin. As the melting point of trilaurin is 43 °C, the extruder barrel was heated to 47 °C which allowed extrusion.

In other experiments, tristearin was substituted by tripalmitin or triarachidin. As H12 was used as the low melting point lipid, the extruder was heated to 40 °C. However, a convenient extrusion procedure could be established only with triarachidin. Substituting tristearin by tripalmitin did not lead to extrudates with the standard process. After filling of the barrel at 40 °C, it was necessary to cool the extruder down to 35 °C to allow extrusion of the lipidic blend. Although numerous lipid blends could be extruded with the described process, further investigations were carried out with extrudates based on H12 and D118.

3.2. Lipid modification after extrusion

The melting of the low melting point lipid during extrusion was essential for the mass flow through the barrel; however this manufacturing strategy may induce a polymorphic transformation to unstable polymorphs. Consequently, a polymorphic transformation to the more stable polymorphs would occur upon storage. Especially in the case of controlled release systems, such a rearrangement might be associated with changes in the matrix structure which would in turn affect the release behaviour [26,35,36]. For example, the preparation of lipid microparticles by the melt dispersion technique or by spraying processes (such as spray congealing or spray drying) resulted in the formation of the α-form [32–34]. This unstable form transformed gradually towards the more stable polymorphs, which was responsible for changes in the appearance and the texture of the microparticles [32,33].

Therefore, the lipid modification of freshly prepared extrudates was investigated by DSC and by WAXS.

First the melting points and the polymorphic behaviour of the bulk lipids H12 and tristearin, respectively, were determined as reference. The thermogram of tristearin revealed one single endothermic transition at 70.8 °C (n=3, SD=1.15 °C), which is characteristic for the melting of the stable β-modification of tristearin (Fig. 1A, black line). Re-crystallisation from melt occurred firstly in the metastable α-form, which is observed in the second heating scan with an endothermic peak at 52.5 °C (n=3, SD=0.75 °C). This endotherm was followed by an exothermic transition representing the re-crystallisation to the β-modification. Upon further heating the melting of the β-modification

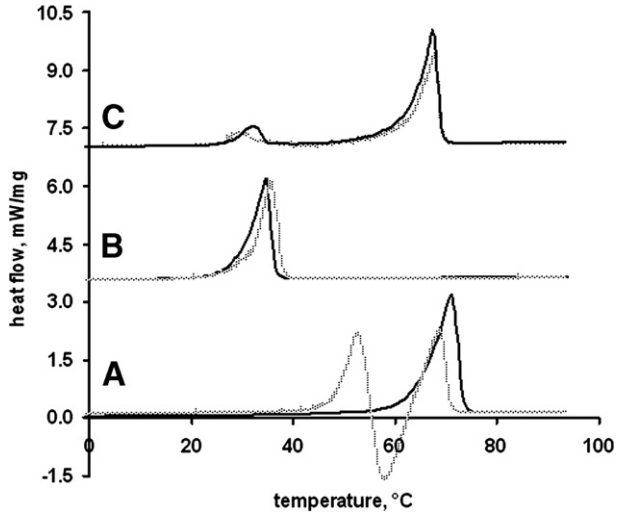

Fig. 1. DSC melting curves of the lipid bulk materials before (black line) and after melting (gray line) (A tristearin, B H12). Part C demonstrates the DSC measurements of the H12/D118 (ratio 1 to 4) lipid blend before extrusion (black line) and after extrusion (gray line). (The plots are displayed vertically for better visualisation.)

was observed at 68.5 °C (n=3, SD=0.75 °C) (Fig. 1A, gray line). The bulk material of H12 revealed one single endothermic peak at 35.6 °C (n=3, SD=0.7; Fig. 1B; black line). After melting and cooling down with liquid nitrogen a similar DSC profile with a melting peak at 35.23 °C (n=3, SD=0.4) is observed (Fig. 1B, gray line).

In the second step the thermal behaviour of the lipidic blend before and after extrusion was investigated. As the melting points of other excipients as for example PEG (T_m=58.7 °C, SD=0.87, n=3) may overlap with the transitions temperatures relevant for studying the polymorphism of the lipids these studies were carried out with formulations consisting solely of lipid material. As shown in Fig. 1C, the lipidic blend before (black line) and after extrusion (gray line) revealed two endothermic transitions. Due to their similarities to the melting temperatures of H12 and tristearin alone the first peak was assigned to the melting of H12 and the second to the melting of tristearin. Compared to the melting temperature of the bulk material both melting events occurred at lower temperatures. Already after grinding and blending a depression in the melting points was observed, that was even more pronounced after extrusion. For instance, H12 bulk material revealed a melting endotherm at 35.6 °C, H12 blended with tristearin featured an endothermic transitions at 32.3 °C (n=3, SD=0.31) prior to extrusion and at 29.82 °C (n=3, SD=0.23) after extrusion. This depression in the melting points together with the fact that the cooling curves after heating revealed two exothermic transitions imply the formation of a eutectic phase rather than the creation of a miscible solid-solution phase. The fact that H12 was completely molten during extrusion presumably explains why the reduction of the melting points is more pronounced after extrusion than after physical mixing.

However, more important for the present work was the observation that no re-crystallisation events or melting peaks of unstable modifications were detectable in the thermograms of freshly prepared extrudates. This clearly indicated that the extrudates comprise both the low- and high-melting point lipid in the stable β-modification.

In order to confirm the absence of instable modifications after extrusion the crystalline state of the lipid bulk materials and lipid extrudates was analysed by wide angle X-ray diffraction (WAXS). As shown in Fig. 2A, the X-ray diffraction patterns of H12 bulk material revealed two strong reflections at 2θ=20.9° d=0.42 nm and at 2θ=23.2° 0.38 nm, which are characteristic for the orthorhombic chain packing of the β' polymorph [37]. Other reflections of lower intensity occurred at 2θ=19.4 d=0.46 nm, 2θ=20.05° d=0.44 nm, 2θ=21.45° d=0.41 nm and 2θ=22.01° d=0.40 nm. After melting and cooling down to room temperature, the diffractogram of H12 still exhibited the typical characteristics of the β′-form with two strong reflections at 2θ=20.9° d=0.42 nm and at 2θ=23.2° 0.38 nm, respectively. In Fig. 2B the WAXS curves of tristearin bulk material are shown. Tristearin featured three dominant diffraction peaks at 2θ=19.5°, 23.3°, 24.3°, respectively. The corresponding short spacings at 0.46 nm, 0.38 nm and 0.37 nm are typical for the β-modification. In contrast, the solidified melt of tristearin showed only one short spacing at about 0.41 nm that can be ascribed to the chain packing of the α-modification [37].

The diffraction patterns of freshly prepared extrudates were compared to the diffraction patterns of the lipidic blend before extrusion. As illustrated in Fig. 3 the mixture of H12 and tristearin produced reflections, which were intermediate between those of the pure substances. The strong diffraction lines at 2θ=19.5°, 23.3°, and 24.3° correspond to the crystal spacings of the β-modification of tristearin at 0.46, 0.38, and 0.37 nm. As pure H12 revealed a diffraction peak at 2θ=23.2° 0.38 nm an overlap with the β′ modification of H12 occurred at this angle. The β′-modification of H12 was detected at 2θ=20.9°0.42 nm. Importantly, the same short spacings were determined after extrusion, leading to the conclusion that tristearin was present in the β-modification and H12 in the β′-modification.

Since both DSC and WAXS analysis of the lipids after extrusion demonstrated that the lipids are present in the stable modification of

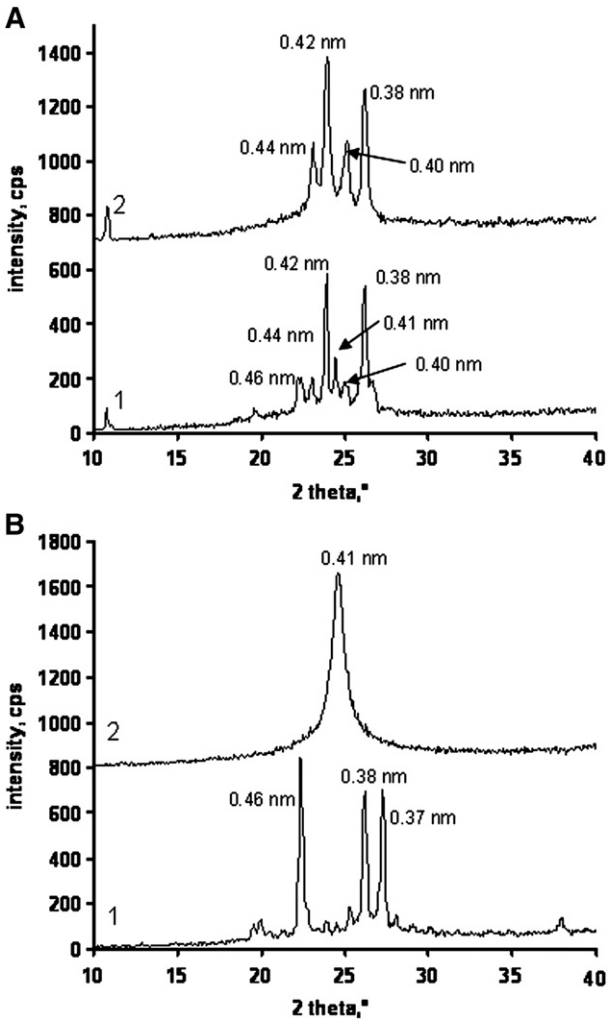

Fig. 2. Diffraction patterns of H12 (A) and tristearin (B). The WAXD diffractograms of the bulk materials and of the molten resolidified lipid are shown in trace 1 and 2, respectively. (The plots are displayed vertically for better visualisation.)

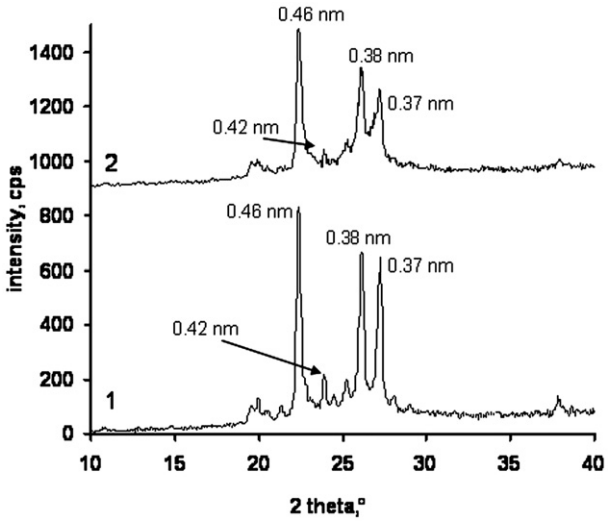

Fig. 3. Influence of the extrusion process on the lipid modification. Diffraction patterns of the H12/D118 (ratio 1 to 4) lipid blend before extrusion (1) and after extrusion (2). (The plots are displayed vertically for better visualisation.)

the bulk materials, unforeseeable and hardly controllable polymorphic transformations upon storage or during release are improbable. The next experiments will, therefore, deal with the second crucial point of the manufacturing procedure: the impact of extrusion on protein stability.

3.3. Influence of the manufacturing process on the protein stability

In order to determine if the extrusion procedure induces protein destabilisation, IFN-α was extracted from the extrudates as previously described [28]. The obtained samples were analysed by SDS-PAGE with subsequent silver staining. In addition to the band arising from monomeric IFN-α, all analysed samples comprised a second protein band with a higher molecular weight (Fig. 4). This dimer fraction was already present in the IFN-α bulk material and in the lyophilisates used for extrusion. IFN-α extracted from the lipidic matrix H12/D118 revealed no further aggregates or fragments.

In addition, conformational analysis of IFN-α embedded within the lipid matrix was performed by Fourier transform infrared (FTIR) spectroscopy. Compared to other analytical methods currently used to characterise proteins after the preparation of controlled release systems, this approach has the advantage that an extraction of the protein from the matrix before analysis is not necessary. Thus, a potential protein denaturation and/or a loss of degraded specimen during the extraction procedure is avoided and the protein structure can be determined quickly within the final product. So far, FTIR spectroscopy was successfully applied to characterise proteins within PLGA microspheres [38–40]. Thereby protein spectra were collected with the KBr pellet technique.

The transmission spectra of the protein-free placebo lipid blend (10% HP-β-CD placebo lyophilisate, 10% PEG, 16% H12 and 64% tristearin), of the triglyceride blend (20% H12 and 80% tristearin) and of the lipid blend loaded with IFN-α (10% IFN-α/HP-β-CD co-lyophilisate, 10% PEG; 16% H12 and 64% tristearin) are shown in Fig. 5. The C=O stretch vibrations of the triglyceride matrix material occurred at 1737, at 1729, and at 1690 cm^{-1}. Protein structure analysis by FTIR spectroscopy is mainly performed by using the amid I absorption region between 1600 and 1700 cm^{-1}. The exact frequencies of amid I absorption can be assigned to particular protein secondary structure elements [41,42]. However, as it can be seen in Fig. 5, the band resulting from carbonyl stretch vibrations of the triglyceride overlaps with the amid I of the protein. Hence, it was necessary to evaluate the possibility of a background correction. These investigations were performed with IFN-α blended in a mortar with the lipidic matrix material. The obtained spectra of the lipid/protein powder blend (10% IFN-α/HP-β-CD co-lyophilisate, 10% PEG, 64% tristearin, and 16% H12) were corrected by the spectra of the respective placebo blend. Yang et al. suggested as a criterion for successful background subtraction a flat baseline in the region at 1730–1710 cm^{-1} [40]. However, as it can be seen in Fig. 6, it was not possible to meet this criterion with the lipidic/protein blend studied. Variations of the subtraction factor either involved an oversubtraction or an undersubtraction of the lipid contributions, leading to negative or positive

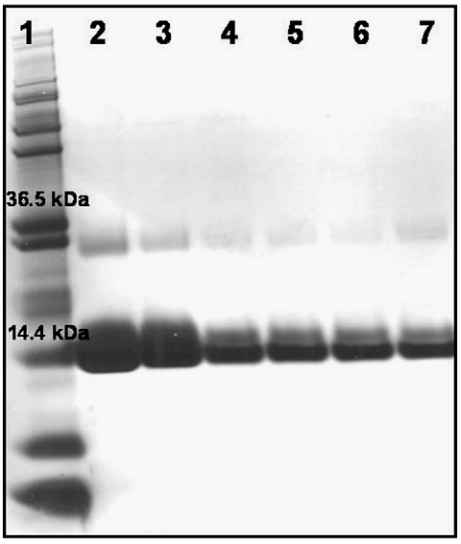

Fig. 4. SDS-PAGE analysis (non-reducing) of IFN-α extracted from lipidic extrudates. Lane 1: Molecular weight standard, lane 2: IFN-α standard, lane 3: IFN-α after lyophilisation, lanes 4–8: IFN-α extracted from lipidic implants. The extrudates formulations were 80% H12/tristearin 1/4, 10% lyophilised IFN-α and 10% PEG (extrudates received at the beginning (lane 4) and at the end (lane 5) of the extrusion procedure, respectively) and 70% H12/tristearin 1/4, 10% lyophilised IFN-α and 20% PEG (extrudates received at the beginning (lane 6) and at the end (lane 7) of the extrusion procedure, respectively).

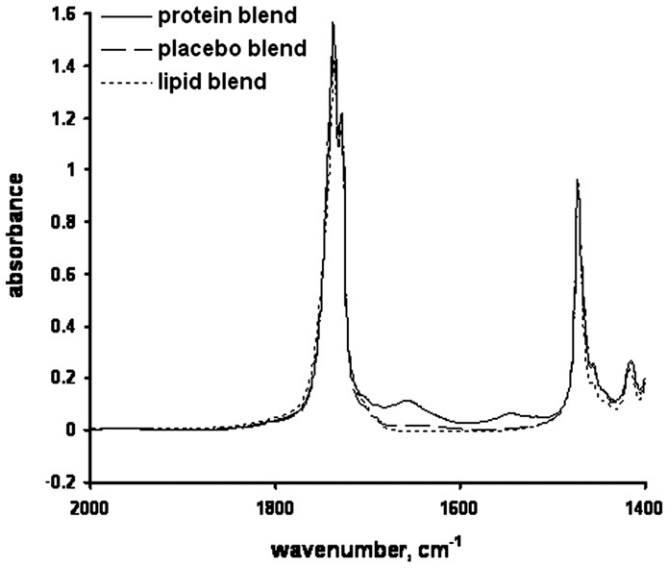

Fig. 5. KBr pellet transmission FTIR-spectrum of the lipid blend (20% H12 and 80% tristearin), of the protein loaded blend ((10% IFN-α/HP-β-CD co-lyophilisate, 10% PEG; 16% H12 and 64% tristearin) before extrusion, and of the placebo lipid blend (10% HP-β-CD placebo lyophilisate, 10% PEG; 16% H12 and 64% tristearin).

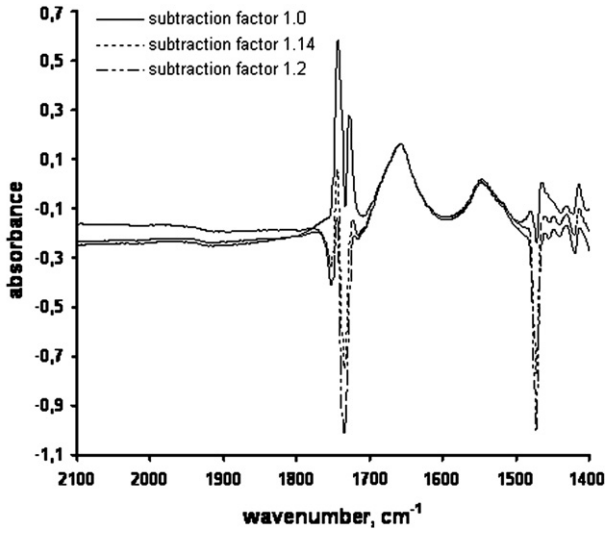

Fig. 6. Effect of background subtraction on the FTIR-spectra. The KBr pellet transmission spectra of IFN-α blended with tristearin, H12 and PEG was corrected by the spectra of the respective placebo blend using different subtraction factors.

baselines in the spectrum between 1700 and 1800 cm^{-1}. The same problem was noted by van de Weert et al. 2000 [39]. They tried to correct the spectra of lysozyme embedded within PLGA microspheres by the spectra of the blank PLGA microspheres. The fact that the background could not be completely cancelled out was explained by baseline slopes and distorted peak shapes especially in the carbonyl stretch vibration of PLGA due to the compression in KBr pellets. In addition, it was suggested that different absorption characteristics of protein-loaded microspheres restricted the subtraction of the spectrum of blank PLGA microspheres [39].

However, in accordance to the work of van de Weert et al. 2000, the peak positions below ~1670 cm^{-1} in the absorbance spectra as well as in the second derivative spectra were not markedly affected by the used subtraction factor (Fig. 7). The obtained spectra were compared with that of lyophilised IFN-α. Both, the spectra of lipid-blended IFN-α after background correction and the reference spectra before blending with the lipid matrix material revealed a dominant band around 1656 cm^{-1}, which is a typical feature of alpha helical proteins [41,42]. Taking together, the similarities of the spectra obtained with different subtraction factors as well as the similarities to the reference spectra of lyophilised IFN-α indicate that the subtraction of the lipid background did not cause spectral artefacts in the amid I region below 1670 cm^{-1}.

In Fig. 8 the second derivative spectra of IFN-α after extrusion with a H12/tristearin blend are illustrated. Compared to the physically blended protein, no significant differences in the amid I region below ~1670 cm^{-1} were detected. Thus, it can be concluded that extrusion of IFN-α embedded in a H12/tristearin blend comprising either 10% or 20% PEG did not induce relevant changes in secondary protein structure.

3.4. In-vitro release studies

The influence of different PEG loadings (e.g.10% and 20%) and the effect of different extrudate diameters on the protein liberation were both studied. For the latter, dies of 0.5 mm or 1 mm were fixed in front of the normal extruder outlet (diameter 2.0 mm). The obtained in vitro release rates are illustrated in Fig. 9. Obviously, the reduction of the extrudate diameter resulted in faster protein delivery. For instance, extrudates comprising 10% PEG liberated IFN-α in a sustained manner over 13 days, when the implant diameter was 0.5 mm. In comparison, the use of extrudates with a diameter of 1.0 or 1.9 mm extended the release period up to 30 or 60 days, respectively. In parallel to this prolongation of the release period, the total amount of IFN-α released differed in dependence on the implant diameter. Almost complete release (95.40% (SD=5.51%, $n=3$)) was determined with the smallest extrudates, whereas increasing the implant diameter resulted in less complete protein release. Extrudates with a diameter of 1.0 mm delivered 83.53% of the incorporated protein within 30 days and a further decrease in the overall released amount was observed with extrudates of a diameter of 1.9 mm (62.09% (SD=0.85%; $n=3$) within 60 days). A similar influence of the extrudate diameter was observed with implants loaded with 20% PEG. Here almost complete protein recovery was observed with extrudates of a diameter of 0.5 and 1 mm. The delivery of 91.18% (SD=0.17, $n=3$) or 93.14% (SD=0.6, $n=3$) IFN-α from these extrudates was retarded over 10 days and over 23 days, respectively. Extrudates with a diameter of 1.9 mm revealed a sustained IFN-α release over 2 months.

The stepwise decrease of the totally liberated IFN-α by increasing the implant diameter indicated that the incomplete protein recovery from larger extrudates can be ascribed to the implant geometry rather than to protein aggregation within the extrudates. When increasing

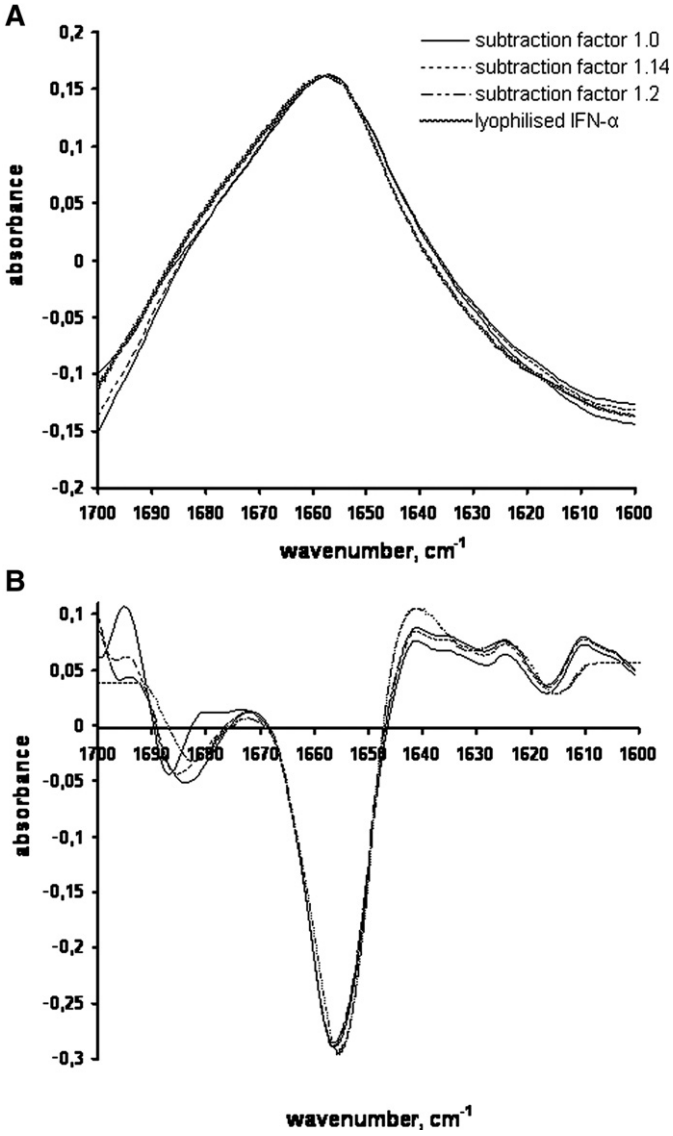

Fig. 7. Vector-normalised KBr-pellet-transmission spectra (A) and respective second derivatives (B) of IFN-α and IFN-α blended with 10% PEG and 80% H12/tristearin 1/4. The background correction of the latter was performed with different subtraction factors resulting in an over- or undersubstraction (compare Fig. 5).

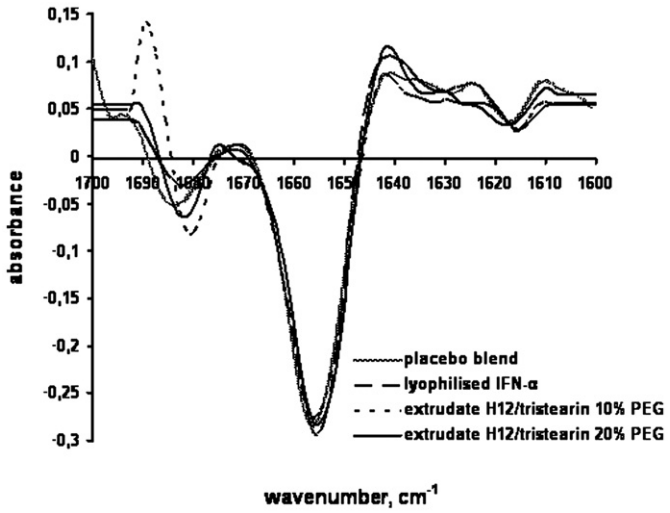

Fig. 8. Influence of the extrusion process on the secondary structure of IFN-α. Second derivative KBr-pellet-transmission spectra of IFN-α after lyophilisation with HP-β-CD, after blending of lyophilisates with PEG, H12 and tristearin, and after extrusion, respectively. For extrusion 10% IFN-α/HP-β-CD co-lyophilisate was blended with 10% or 20% PEG and 80% or 70% H12/tristearin 1/4.

the diameter of extrudates the diffusion pathways increase, therefore, it seems that the enlargement of the matrix requires more hydrophilic drug/excipient to create a connected pore network.

Beside the adaptation of the release kinetics by using extrudates with different diameters, the addition of various amounts of PEG was an effective tool to modify IFN-α release. Higher amounts of incorporated PEG resulted in a more accelerated IFN-α release. For example extrudates with a diameter of 1.0 mm comprising 10% PEG liberated IFN-α in a sustained manner over 37 days, whereas the delivery was only retarded over 19 days when 20% PEG was used as pore former.

Interestingly, the release kinetics of extrudates with a diameter of 1.9 mm (partly also extrudates with a diameter of 1.0 mm) revealed a lag-phase after the initial burst release. Extrudates loaded with 10% PEG released 10.41% (SD=2.02%, $n=3$) within the first day of incubation. After that, a lag-phase of about 15 days followed. Increasing the amount of admixed PEG to 20% provoked a burst release of 7.19% (SD=0.65%, $n=3$) and shortened the lag-phase to 7 days (Fig. 9C). After the lag-period IFN-α was delivered from extrudates with 10% PEG in a constant manner over 30 days until day 53 of incubation. In comparison to that, extrudates with 20% PEG revealed a linear delivery phase over 24 days. Such a triphasic release profile (burst, lag-phase, linear release period) was often reported for protein delivery from degradable matrix systems. Thereby, the burst is ascribed to protein delivery from the surface. The lag-phase is characterised by diffusion controlled release and finally, when erosion starts, the release rates increase [43–45]. However, extrudates based on a H12/tristearin blend maintained their geometric dimensions during in vitro incubation, and no mass loss occurred during incubation [judged by visible inspection of the extrudates and on the weight of the extrudates after in-vitro incubation]. Consequently, the mechanisms for a triphasic release profile must be different. It can be assumed that the previously reported in-situ precipitation of IFN-α within PEG-containing lipidic implants [46,47] might account for the observed release profile: When we suppose high concentrations of PEG within the implant pores after the initial burst period, an extremely low solubility of IFN-α can be expected, explaining the observed non-release phase. Afterwards, due to the release of PEG, the solubility of IFN-α and thus the release rates increase. Even though detailed mechanistic studies were beyond the scope of this study, the described explanation can be backed by the observation that extrudates with a diameter of 1.9 mm revealed a similar burst release when the PEG content was increased from 10% to 20%. Such an observation contradicts to the effects of PEG as a porogen [20,48,49], but was shown to be a result of the postulated in-situ precipitation mechanism reported for PEG-containing compressed tristearin matrices before [46,47].

Finally, a controlled release device can only be considered as appropriate if the delivery of native protein specimen is ensured. Protein degradation products might show less or no bioactivity, which will imperil the therapeutic efficiency of the product. Moreover, the delivery of non-native protein and especially protein aggregates risks the induction of an immune response [50,51]. Though bioactivity testing of the released protein must be considered as the final proof of the capability of lipidic extrudates as delivery system for proteins, SE-HPLC analysis of released IFN-α was carried at least to ensure the

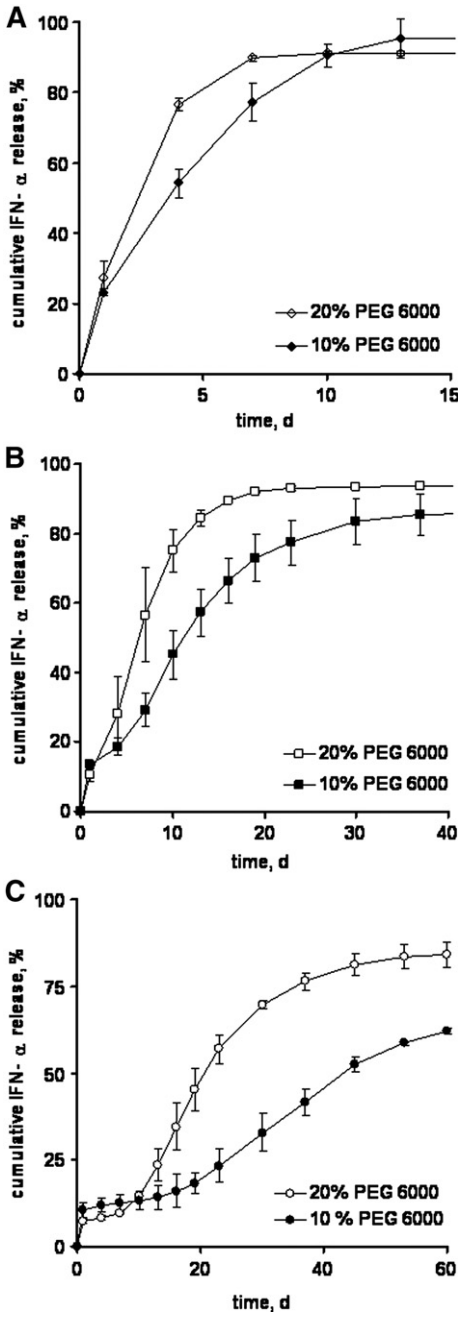

Fig. 9. The effect of different PEG loadings and different implant diameters on the in-vitro release behaviour of IFN-α from lipid extrudates. For extrusion 10% IFN-α/HP-β-CD co-lyophilisate was blended with 10% or 20% PEG and 80% or 70% H12/tristearin 1/4. Extrusion was performed with the described twin screw extruder at 40 °C. The diameter of the prepared rods was 0.5 mm (A), 1.0 mm (B), or 1.9 mm (C) (average+/−SD; $n=3$).

absence of high molecular weight degradation products. Importantly, IFN-α was mainly delivered as monomer. Over the entire liberation period, the IFN-α monomer content remained at a high level (>95%) and only dimer specimen were detected by SE-HPLC.

4. Conclusion

In this work extrusion was investigated as an alternative manufacturing technique for the preparation of controlled release systems for pharmaceutical proteins. Using a combination of a low melting point and high melting point triglycerides, extrusion could be realised at moderate temperatures. It was shown that the developed extrusion protocol neither affects the modification of the lipids nor the stability of the incorporated model protein IFN-α. The protein structure was thereby not only investigated by gel electrophoresis after extraction from the matrix but also within the extruded implant by FTIR-spectroscopy. Protein release from the developed extrudates can easily be controlled by variation in implant diameter or by the admixing of various amounts of PEG. For instance, protein liberation could be controlled in a prolonged manner over 15, 40, or 60 days by producing extrudates with a diameter of 0.5 mm, 1.0 mm or 1.9 mm, respectively.

Apart from these promising results showing the potential of the extruded implants for the sustained release of proteins, the system offers the benefit that manufacturing by twin-screw extrusion can be easily scaled up. Furthermore, the geometry of the implants, in particular their small diameter, would allow subcutaneous injection via a large gauge needle.

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